

## REVIEW

# Mass spectrometry-based proteomics for the detection of plant pathogens

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Plant diseases caused by fungi, oomycetes, viruses, and bacteria are devastating both to the economy and to the food supply of a nation. Therefore, the development of new, rapid methods to identify these pathogens is a highly important area of research that is of international concern. MS-based proteomics has become a powerful and increasingly popular approach to not only identify these pathogens, but also to better understand their biology. However, there is a distinction between identifying a pathogen protein and identifying a pathogen based upon the detection of one of its proteins and this must be considered before the general application of MS for plant pathogen detection is made. There has been a recent push in the proteomics community to make data from large-scale proteomics experiments publicly available in the form of a centralized repository. Such a resource could enable the use of MS as a universal plant pathogen detection technology.

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## 1 Introduction

Even though scientific methods are applied for pathogen detection, and the rigorous Koch's postulates are still followed for the proof that a detected pathogen causes disease, the correct diagnosis of a plant disease and the subsequent detection or identification of the disease causing agent is still an art form. While host range tests and symptom evaluation are some of the best ways to characterize plant pathogens, these methods are time-consuming and require patience and a good eye for observing symptoms. Fortunately, technology advancements have brought more science to the practice of the art. Unfortunately, technologies have their faults. Microscopy is an excellent method for evaluating the morphological characteristics of a broad spectrum of pathogenic organisms, but it is not sufficient for identifying some patho-

gens or specific species that cannot be distinguished visually. By comparison, nucleic acid hybridization, PCR, DNA sequencing, dsRNA analysis, and antibody-based methods (ELISA and Western blots) are scientific methods that have the unique ability to define strains at a molecular and genetic level [1, 2]. Many of these methods are easy to perform, and are inexpensive, fast, and precise. However, they do require pathogen-specific reagents, meaning that these methods are not as useful if the reagents are not available. Because no single method is always reliable or available, plant pathologists routinely use a combination of both science and art forms to identify pathogens. Despite better attempts, any plant pathologist who has spent several months' tedious analysis trying to identify a pathogen has wished for one technology that could supplant the many. MS holds such a promise. With its ability to discriminate protein and peptide masses that are unique to pathogens without the requirement of pathogen-specific reagents to first perform the analysis, MS has the potential to be a universal pathogen detection technology that rivals DNA sequencing.

As a diagnostic practice, there is very little current application of MS for plant pathogen detection and our extensive survey of literature while assembling this report proved this to be true. Plant pathologists are not, however, ignorant of MS, but they tend to be using it to study proteome changes of the plant to gain

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**Abbreviations:** CP, capsid protein; CSI, cross-species identification; JGMV, Johnsongrass mosaic virus; TMV, tobacco mosaic virus

insight into the biology of host responses to infection. Although measuring host responses to infection could be viewed as one component of disease diagnosis, disease diagnosis and pathogen detection are not necessarily the same concepts. Therefore, it is best to accept that in most cases evaluating the host is not as favorable as detecting the pathogen directly. Nevertheless, for a review of this type, it is worthwhile to peruse the literature to see if researchers have detected pathogen proteins while evaluating infected hosts, since this parallels the scenario of a technician diagnosing a diseased plant leaf and trying to find pathogen proteins among plant proteins. In a cursory examination, we looked at nine recently published manuscripts where the authors used MS to study the proteomics of a plant's response to a pathogen (Table 1). All report a number of different plant proteins that accumulate as a result of infection, but none reports finding or detecting any pathogen proteins. Why? Were pathogen proteins detected but just not reported? We find this strange since it seems like it would be good practice to show as a positive control that the plants were indeed infected with the pathogen. Is something else at hand? Are the pathogen proteins unresolvable because of lack of information on the mass or sequence of the pathogen proteins? Or is the technology not sensitive enough to differentially resolve or detect pathogen proteins that might not be in abundance in relation to some of the more abundant plant proteins? Thus, in deference to these questions, we turn our attention to the few reports whereby plant pathogen proteins have been detected by MS and begin our discussion on the merits of using MS for plant pathogen detection.

## 2 MS and the identification of viral plant pathogen proteins

As a result of its vast range of applications, MS has proved its use for the identification of viruses better than for any other pathogen. MS methods for determining the identity of a plant virus

can be broadly placed into the following categories: (i) identification based on the total mass of the viral capsid protein (CP); (ii) identification based on using the masses of proteolytic peptides from the CP as a unique fingerprint (also known as PMF); and (iii) resolution of amino acid sequences of CP peptides *via* MS/MS. Key studies that illustrate the use of these various methods to identify plant viruses are highlighted below.

In one of the first examples of using MS to detect plant viruses, Fenselau and coworkers [3] analyzed infected tobacco leaves by MALDI-TOF MS directly after the addition of acetic acid and matrix solution. The MALDI mass spectrum contained a single peak at 17 491 Da, which corresponded to the known mass of the tobacco mosaic virus (TMV) CP. Using a similar approach, Tan *et al.* [4] used MALDI-TOF MS and LC/MS to simultaneously identify CPs of two viruses from an infected orchid. The known molecular masses of the corresponding CPs also enabled these findings. These examples clearly point to the very suitable diagnostic benefits of identifying viral CPs by known masses. However, this diagnostic practice suffers if the mass of the virus CP is unknown or if there is variance between the predicted mass from a nucleic acid sequence and the observed mass. Unpredictable genetic mutations or PTMs such as glycosylation and phosphorylation can create discrepancies that confound the interpretation of data and the accurate identification of the virus [5–7]. In such cases, high resolution MS is particularly amenable to addressing this problem and MALDI MS has been used to reliably identify mutations in the TMV CP [8]. Compared to DNA sequencing that can be used to identify the origin of protein variation, only MS can resolve the same mutations and any PTMs, which can become an important distinction with regard to pathogen detection by virtue of protein identification.

When mass alone is not sufficient for identifying a CP, an additional level of interrogation can be added by digesting the CP with a protease and examining the masses of the derivative peptides. By manually examining the masses of

**Table 1.** Recent reports on plants infected with pathogens and the use of MS to identify proteins

Manuscript	Plant/pathogen	MS method	No. of plant proteins reported	No. of pathogen proteins reported
Ndimba <i>et al.</i> [67]	<i>Arabidopsis thaliana</i> / <i>Fusarium moniliforme</i>	MALDI-TOF MS	8	0
Jones <i>et al.</i> [68]	<i>A. thaliana</i> / <i>Pseudomonas syringae</i> pv. Tomato DC3000	LC-QTOF MS/MS	7	0
Subramanian <i>et al.</i> [69]	<i>Brassica napus</i> , <i>B. carinata</i> / <i>Leptosphaeria maculans</i>	MS/MS	40	0
Campo <i>et al.</i> [70]	<i>Zea mays</i> / <i>F. verticillioides</i>	MALDI-TOF MS	17	0
Rep <i>et al.</i> [71]	<i>Lycopersicon esculentum</i> / <i>F. oxysporum</i>	nanoESI-IT MS/MS		
		MALDI-TOF MS	17	0
		nanoESI-QTOF MS/MS		
Colditz <i>et al.</i> [72]	<i>Medicago truncatula</i> / <i>Aphanomyces euteiches</i>	MALDI-TOF MS	12	0
Ventelon-Debout <i>et al.</i> [73]	<i>Oryza sativa</i> /RYMV	MALDI-TOF/TOF MS	64	0
		nanoLC-QTOF MS/MS		
Kim <i>et al.</i> [74]	<i>O. sativa</i> / <i>Magnaporthe grisea</i>	MALDI-TOF MS	8	0
Smith <i>et al.</i> [75]	<i>Pinus strobus</i> / <i>Cronartium ribicola</i>	MS/MS	11	0

their tryptic digestion products, She *et al.* [9, 10] successfully resolved the amino acid sequences of several brome mosaic virus isolate CPs and also discovered the true amino acid sequence of the CP of high plains virus, whose genome sequence was incomplete by comparison. These studies also prove the utility of MS in virus identification. However, the ability to manually interpret the masses of digestion products is beyond the routine capabilities of most plant pathogen diagnostic laboratories. Fortunately, it is possible to automate the evaluation of mass spectra by using computer algorithms that compare observed PMFs to virtual fingerprints from the known sequences of candidate viruses [11]. With a little training, it is possible for many laboratories to identify CPs of plant viruses based on PMFs, even if the masses have been altered by PTMs since potential mass modifications can be considered by the algorithms.

A third level of interrogation can be incorporated by performing MS/MS to resolve the amino acid sequences of tryptic peptides. As shown by Cooper *et al.* [12], 2-DE was used as a separation method to differentiate between proteins from infected plants and noninfected plants, and MS/MS spectra of peptides from candidate viral proteins were produced. These authors then relied on a database search algorithm to interpret the MS/MS data and produce a peptide sequence inference for the tandem mass spectra based on known viral CP sequences from a protein sequence database. Consequently, they identified two viruses from infected plants, one of which was an unknown that had not been detected by using a standard set of PCR primers. It is worth noting that had the virus CP sequence not been in the search database, the identity of the unknown virus might have remained unknown. These potential shortcomings of using peptide and protein sequence databases to decipher tandem mass spectra will be discussed in the next section.

At the very least, MS is useful for obtaining complementary identification information when data from other methods is not sufficient. For example, Seifers *et al.* [13] observed a virus causing new symptoms in sorghum, a virus that weakly reacted with Johnsongrass mosaic virus (JGMV) antiserum. MS/MS experiments on the CP of the new virus revealed that the intermediate region of the CP was substantially different from the corresponding region in the CP of the virus to which the antiserum was raised. Consequently, these authors were able to characterize a new JGMV strain and explain the weak interactions of the antiserum. Certainly, because of the utility of MS and different levels of interrogation that can be performed on proteins, there will likely be many more instances whereby MS is used in conjunction with other molecular methods to identify viruses.

### 3 MS and the identification of fungal and oomycete plant pathogen proteins

Plant pathogenic fungi cause an enormous amount of damage to important crops all around the world. These fungal pathogens are very diverse and the gene sequences of many

of these fungal pathogens are not well characterized. Thus, there are few protein sequence references available to reliably use MS to detect fungal proteins with the same ease and accuracy as plant viral proteins. The precariousness of the lack of references is apparent in several recent proteomic investigations of fungi. For example, when performing MS analysis on proteins from the biocontrol fungus, *Trichoderma harzianum*, only five proteins from *Trichoderma* spp. were detected, mainly because only a few *Trichoderma* spp. protein sequences existed in the search databases [14]. To try to get a better understanding of more *T. harzianum* proteins, cross-species identification (CSI) was used to compare the PMFs against all of the known protein sequences in the fungal kingdom. CSI is based on the partial alignment of a protein from an organism with an unsequenced genome to a characterized protein from a related organism whose sequence is known [15–17]. In this case, matches were made to proteins from related Ascomycetes such as *Neurospora crassa* and *Aspergillus oryzae*. Insight was gained on the function of these likely homologous proteins in *T. harzianum*, but it is important to reiterate that *T. harzianum*-specific sequences from the database were not used for these matches.

Similarly, CSI aided the identification of proteins from spores of the common bean rust fungus *Uromyces appendiculatus* [18]. Whereas only three identifications were made to known *Uromyces* spp. proteins, a number of other proteins were identified based on peptide sequence inferences made from proteins from other fungi, including 25 proteins from *Ustilago maydis*, a related Basidiomycete plant pathogenic fungus whose genome is sequenced. From both of these examples, it is apparent that the approaches are valid for unraveling the potential function of proteins and elucidating the fungal biology of *T. harzianum* and *U. appendiculatus*. But in the context of pathogen detection, it seems that CSI PMF matches and CSI peptide sequence inferences could complicate an attempt to identify a pathogen by MS. A simple thought experiment makes it clear: Which fungus from an unknown sample is identified if a technician matches three proteins to *Uromyces* spp. and 25 proteins to *Ustilago* spp. using MS/MS? Had the sample come from *U. appendiculatus* in the previous example, an assumption that *U. maydis* was identified because more *Ustilago* spp. proteins were used for the inferences would be wrong. Complicate the scenario with a background of plant proteins, a possible mixed infection, or the presence of beneficial fungi such as *Trichoderma* spp. and it becomes apparent that it may not be possible to distinguish plant from pathogen or pathogen from other fungi without more comprehensive protein sequence databases.

There are three other complications with fungal protein identification by MS that can impede its use for fungal pathogen detection. First, unpredictable PTMs can be produced by both the fungus and the host which could possibly confound PMF matches or peptide sequence inferences. Second, the issue of protein detection is far more complex for fungi by comparison to the previously noted virus examples. Plant viruses encode four to ten proteins whereas a plant pathogenic fungus can encode several thousands of proteins.

In addition, viral CPs can comprise 1–3% of the total soluble protein in a plant cell [19]. For fungi, it is likely that no single fungal protein will accumulate to such high levels by comparison, especially with regard to the thousands of other proteins that are required for the routine cellular biology of the fungus. Couple protein diversity with low accumulation and it becomes clear that it may be challenging to resolve the required proteins for fungus detection, especially with respect to a complex plant protein background. Third, fungi can have several life cycle stages, which means that the presence of some proteins that are used in identification can be in flux. There is considerable interest in identifying which proteins are specific to the various stages of fungal development. Recent studies with *Phytophthora palmivora* [20] and *Phytophthora infestans* [21] demonstrate that MS-based proteomics is an effective strategy to identify proteins that are specific to various growth stages. Nevertheless, these three factors must be considered when trying to identify fungi and similar pathogens with complex lifecycles.

#### 4 MS and the identification of bacterial plant pathogen proteins

The remarkable speed of data acquisition makes the application of MS to identify and characterize bacteria principally appealing. In this context, MS is used primarily as a fingerprinting tool in order to acquire unique signatures of a bacterium. For quite some time, the basis of these signatures has been small molecule components in bacteria such as lipids, phospholipids, carbohydrates, and secondary metabolites, but limited success has been achieved in terms of developing reliable biomarkers based on these small molecule components [22]. The development of MALDI [23] and ESI [24] MS has caused many researchers to shift their attention to proteins for bacterial identification due to their abundance in the cell along with the fact that they confer indirect genetic information pertaining to the bacterium under investigation. The analysis of bacteria by MS has become a mature field and many excellent reviews have been written that detail the various techniques involved in the characterization of the proteomes of bacteria [22, 25, 26].

Few bacterial plant pathogens have sequenced genomes, thus many of the same problems associated with performing diagnostic MS on fungi apply to bacteria. Because most bacterial plant pathogens are not genetically well characterized and only a small handful of complete genome sequences exist for these pathogens, approaches that have been developed to characterize bacteria based on searching MS/MS data of tryptic peptides against publicly available protein databases have very little utility in terms of being able to identify bacterial plant pathogens. Nonetheless, Dworzanski *et al.* [27] reported the development of a method to classify bacteria based on data from LC-MS/MS experiments. This method can classify bacteria to the strain level if a sufficient amount of sequence information is obtained from the MS/

MS experiment. Since this method also relies on searching a protein sequence database, it also requires a genome/proteome sequence for the organism and suffers the same limitations as already noted. However, this method may have great utility for a wide range of bacterial plant pathogens in contrast to fungi since a greater number of bacterial genomes will be sequenced over time by comparison due to the relative ease of sequencing small genomes.

#### 5 On peptide sequence inferences and the development of biomarker libraries

The power of MS/MS for pathogen protein identification is the potential to resolve the amino acid sequence of a protein in addition to determining its mass. Top-down proteomics and expert manual interpretation of mass spectra has clearly achieved the most accurate peptide sequences for the identification of some bacterial pathogen proteins [28]. Unfortunately, the ability to perform such analyses is limited to only the best MS groups in the world. Therefore, plant pathologists/budding mass spectrometrists will likely rely on some type of computer algorithm to interpret mass spectral data. Thus, comprehensive detection capabilities will require more pathogen-specific protein information for the databases. Already, MS has aided in the acquisition of such information. Rep *et al.* [29] used MS/MS to identify novel peptides from a 12 kDa cysteine-rich protein that is secreted by the fungal pathogen *Fusarium oxysporum* in the xylem vessels of the tomato plant during infection. They designed a set of degenerate primers from sequences deduced from spectra and were able to amplify a corresponding clone from a cDNA library which had very little similarity to any other known protein [29, 30]. Similarly, Apoga *et al.* [31] applied 2-DE and MS/MS to identify novel biotinylated/affinity-purified proteins in the extracellular matrix of the plant pathogenic fungus *Bipolaris sorokiniana*. Others have revealed similar insight into the molecular basis of pathogenicity from proteomic investigations of *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot disease in crucifers [32], *Xylella fastidiosa*, the causal agent of citrus variegated chlorosis [33], and *Erwinia chrysanthemi*, the causal agent of soft rot disease on many different plants [34]. All of these studies have led to the discovery of many different proteins that confer unique pathogenic characteristics to these pathogens. It is possible that proteins from these studies could be used as unique identifiers for these organisms, and the results certainly point to the promise of using MS to identify unique proteins that can be deposited in a searchable database. Future investigators who unambiguously resolve these unique proteins will certainly have to consider the possibility that these organisms were detected.

Despite the fact that databases will become more comprehensive over time, allowing for more pronounced peptide identifications across a wide range of organisms, it has become very apparent over the last few years that peptide

sequence inferences from database searches are associated with a number of problems, problems that also directly translate to the identification of associated proteins [35]. One such problem, distraction, the phenomenon whereby the appearance of false-positive inferences increases disproportionately as the database size grows [36, 37], can possibly be countered with improved mass accuracy [38, 39]. Improved statistical and probability measures can also be incorporated to give greater confidence in peptide sequence inferences and protein identification [35, 40]. However, peptide inferences are susceptible to change as search databases evolve and there will always be uncertainty regarding pathogen identification and disease diagnosis if detection is purely based on peptide sequence inferences of tandem mass spectral data. Notwithstanding, some peptides are distinct to a particular organism and hence, the tandem mass spectra of these peptides are also unique signatures that can be used to identify an organism [41, 42]. In other words, peptide tandem mass spectra themselves are suited for being biomarkers for an organism since the spectral information remains static, even as protein sequences in databases change. It is likely that peptide tandem mass spectral biomarkers are better representative signatures for an organism than DNA sequence, since they reflect important modifications to the proteins and peptides that cannot be readily predicted from the DNA sequence alone.

One argument for using peptide tandem mass spectra directly as biomarkers rather than glean peptide sequence information from them is that the majority of peptide tandem mass spectra that are acquired in a typical shotgun proteomics experiment are not assigned to a peptide sequence and hence, are not further analyzed [43]. This can be explained by incomplete databases, deficiencies in the algorithms that make peptide sequence inferences, or variations in the proteins that arise from mutation, splice variation or chemical or PTM. In short, valuable peptide information that could be used to identify an organism is not efficiently utilized. Removing the artificial layer of interpretation of peptide sequence inferences allows for the comparison of the true signature of a peptide, the tandem mass spectrum. Evaluated as whole units, it is possible that the matching of peptides based on spectrum-to-spectrum comparisons can likely identify peptides at higher levels of confidence than methods based on sequence information only [44].

Consequently, peptide tandem mass spectral biomarker libraries can be created for plant pathogenic organisms and subsequently searched for matching spectra. Matches would be used to confirm the presence of spectra distinguishing a pathogen. Similar approaches of mass spectral library searching [45–47] are extensively and successfully applied for the identification of pesticides [48], flavors and fragrances [49], volatile organic compounds [50], and metabolites [51–55]. In fact, the use of PMFs to identify bacterial proteins from a searchable PMF database is just another form of mass spectral library searching [56, 57] and recent strides in MS spectral comparison algorithms makes PMF biomarker searching for protein identifica-

tion more reliable for plant pathologists who rely extensively on PMF to characterize gel-separated proteins [58]. Nonetheless, whereas PMF biomarkers can enable pathogen protein identification, peptide tandem mass spectral biomarkers have an advantage over PMFs because the peptide tandem mass spectra contain amino acid sequence information that can also be used in other downstream protein characterization analyses.

There has been a recent push to make tandem mass spectral libraries from large-scale proteomics experiments available to the scientific community in the form of a centralized repository such as the Open Proteomics Database [59], Peptide Atlas [60], and PRIDE [61]. Plant pathologists would benefit from such a repository containing unique plant pathogen peptide tandem mass spectral biomarkers. Once a set of standards is established, it should be easy for capable laboratories to create peptide tandem mass spectral biomarkers for a wide variety of plant pathogens, especially by contrast to the relative difficulty of acquiring an equivalent amount of genome information for the same number of organisms. Libraries would be assembled by clustering spectral data, first by evaluating a suitable similarity measure between two tandem mass spectra (*e.g.*, normalized dot product, Euclidian distance, *etc.*) followed by clustering very similar spectra and producing a representative spectrum for the cluster [62]. Additional information could be considered to improve cluster creation, such as retention time [63] and a number of different criteria could be imposed to filter out unworthy spectra [64]. Finally, the same algorithms that were used to develop the libraries in the first place could then be used for their subsequent searching. To aid comparisons, libraries could be developed to contain a wide-range of possible tandem mass spectra contaminants and software would apply rigorous statistical measures to help reduce false-positive findings and increase the confidence and probability of finding a true match to the database. A centralized repository for tandem mass spectra of peptides derived from plant pathogens could be maintained. The National Institute of Standards and Technology has created similar mass spectral databases for small molecules and is well-recognized for providing this valuable resource to scientists around the world. The hosting of a pathogen peptide mass spectra library could similarly provide great opportunities to a number of interested institutions.

## 6 Summary

The accidental or deliberate release of a plant pathogen could result in a major devastation to both the economy and food supply of a nation. The unresolved release of anthrax that followed the events of September 11, 2001 served as a stark reminder that the unlawful dissemination of a biological agent is a real threat and heightens biosecurity measures are greatly needed [65]. With international travel on the rise, the possibility of exotic pests and pathogens entering the country is now greater than ever before [1]. Changes to the global

weather also appear to be influencing pathogen spread; *Phakopsora pachyrhizi*, the causal agent of soybean rust, likely entered the US from South America from the remnants of Hurricane Ivan in September 2004 [66]. As the world seems to get smaller, there appears to be ever increasing possibilities for pathogen movement. Rapid identification is perhaps the best defense against the introduction of new diseases into the country.

MS-based proteomics will become a critical approach to identify, characterize, and better understand the biology of fungal, oomycete, viral, and bacterial plant pathogens. The lack of genome information available for these pathogens does continue to be a bottleneck to their successful identification when using MS. This may be one reason why some researchers have not reported the detection of pathogen proteins when performing proteomics on infected plants. For the same reason then, there is justification to avoid using peptide sequence inferences for the broad application of MS for pathogen identification. Since peptide sequence inferences are really a reflection of the maturation of DNA sequencing technologies and databases, we believe that the creation and searching of dedicated peptide tandem mass spectra biomarker libraries is actually truer to the potential advantages of MS technology for pathogen identification.

With the exception of just a handful of documents showing that MS has been used for plant pathogen identification, we are unaware of any practical use of MS in plant pathology laboratories for pathogen detection. This truly is an infant technology for an established and entrenched field. Before wide-scale use of MS for plant pathogen detection is deployed or accepted by the plant pathology community at large, a number of experiments must be performed. A broader MS survey of plant pathogens will be required, especially if peptide tandem mass spectral databases are to be curated. With this mind, we are currently trying to identify proteins from several fungal plant pathogens such as *U. maydis*, *Fusarium graminearum*, and *Rhizoctonia solani*. At some point, MS must also be directly compared to other technologies before it can be efficiently and confidently used for detection. Will MS have the capability of detecting plant pathogens when other methods are insufficient? Yes and this has been already shown [12]. Will MS supplant the cost-effectiveness or speed of PCR or ELISA if pathogen-specific reagents are available? We doubt it. Will MS be as sensitive as PCR? Time will tell but MS can detect single ionized peptides just as readily as PCR can enable the detection of single DNA molecules through amplification. In the end, we expect that plant pathologists will add MS to their quivers and will arm themselves with the technique as needed or use it in combination with other identification and detection methods when trying to target a diagnosis.

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